Depatuxizumab Mafodotin (ABT-414)-induced Glioblastoma Cell Death Requires EGFR Overexpression, but not EGFR Y1068 Phosphorylation

von Achenbach, Caroline ; Silginer, Manuela ; Blot, Vincent ; Weiss, William A ; Weller, Michael

Abstract: Glioblastomas commonly (40%) exhibit epidermal growth factor receptor (EGFR) amplification; half of these tumors carry the EGFRvIII deletion variant characterized by an in-frame deletion of exons 2-7, resulting in constitutive EGFR activation. Although EGFR tyrosine kinase inhibitors had only modest effects in glioblastoma, novel therapeutic agents targeting amplified EGFR or EGFRvIII continue to be developed.Depatuxizumab mafodotin (ABT-414) is an EGFR-targeting antibody-drug conjugate consisting of the mAb 806 and a toxic payload, monomethyl auristatin F. Because glioma cell lines and patient-derived glioma-initiating cell models expressed too little EGFR in vitro to be ABT-414-sensitive, we generated glioma sublines overexpressing EGFR or EGFRvIII to explore determinants of ABT-414-induced cell death.Overexpression of EGFRvIII induces sensitization to ABT-414 more readily than overexpression of EGFR in vitro and in vivo Exposure to ABT-414 in vivo eliminated EGFRvIII-expressing tumor cells, and recurrent tumors were devoid of EGFRvIII expression. There is no bystander killing of cells devoid of EGFR expression. Surprisingly, either exposure to EGF or to EGFR tyrosin kinase inhibitors reduce EGFR protein levels and are thus not strategies to promote ABT-414-induced cell killing. Furthermore, glioma cells overexpressing kinase-dead EGFR or EGFRvIII retain binding of mAb 806 and sensitivity to ABT-414, allowing to dissociate EGFR phosphorylation from the emergence of the ”active” EGFR conformation required for ABT-414 binding.The combination of EGFR-targeting antibody-drug conjugates with EGFR tyrosine kinase inhibitors carries a high risk of failure. Promoting EGFR expression rather than phosphorylation should result in glioblastoma cell sensitization to ABT-414.

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Depatuxizumab mafodotin (ABT-414)-induced glioblastoma cell death requires EGFR overexpression, but not EGFR^{Y1068} phosphorylation

Caroline von Achenbach[1], Manuela Silginer[1], Vincent Blot[2], William A. Weiss[3] and Michael Weller[1]

[1]Laboratory of Molecular Neuro-Oncology, Department of Neurology, University Hospital and University of Zurich, Zurich, Switzerland

[2]Abbvie Inc., North Chicago, IL

[3]Departments of Neurology, Pediatrics, Neurosurgery, Brain Tumor Research Center, and Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco, CA

Corresponding author: Michael Weller, MD, Laboratory of Molecular Neuro-Oncology, Department of Neurology, University Hospital Zurich, Frauenklinikstrasse 26, 8091 Zurich, Switzerland, Tel.: +41 44 255 5500, E-mail: michael.weller@usz.ch

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Conflict of Interest

CvA and MS report no conflicts of interest.

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Abstract

Glioblastomas commonly (40%) exhibit epidermal growth factor receptor (*EGFR)* amplification; half of these tumors carry the EGFRvIII deletion variant characterized by an in-frame deletion of exons 2-7, resulting in constitutive EGFR activation. Although EGFR tyrosine kinase inhibitors had only modest effects in glioblastoma, novel therapeutic agents targeting amplified EGFR or EGFRvIII continue to be developed.

Depatuxizumab mafodotin (ABT-414) is an EGFR-targeting antibody drug conjugate consisting of the monoclonal antibody 806 and a toxic payload, monomethyl auristatin F. Since glioma cell lines and patient-derived glioma-initiating cell models expressed too little EGFR *in vitro* to be ABT-414-sensitive, we generated glioma sublines overexpressing EGFR or EGFRvIII to explore determinants of ABT-414-induced cell death.

Overexpression of EGFRvIII induces sensitization to ABT-414 more readily than overexpression of EGFR *in vitro* and *in vivo*. Exposure to ABT-414 *in vivo* eliminated EGFRvIII-expressing tumor cells, and recurrent tumors were devoid of EGFRvIII expression. There is no bystander killing of cells devoid of EGFR expression. Surprisingly, either exposure to EGF or to EGFR tyrosin kinase inhibitors reduce EGFR protein levels and are thus not strategies to promote ABT-414-induced cell killing. Furthermore, glioma cells overexpressing kinase-dead EGFR or EGFRvIII retain binding of mAb 806 and sensitivity to ABT-414, allowing to dissociate EGFR phosphorylation from the emergence of the “active” EGFR conformation required for ABT-414 binding.

The combination of EGFR-targeting antibody drug conjugates with EGFR tyrosine kinase inhibitors carries a high risk of failure. Promoting EGFR expression rather than phosphorylation should result in glioblastoma cell sensitization to ABT-414.

Keywords

ABT-414, amplification, ADC, EGFR, glioma

Introduction

Glioblastoma is an invariably fatal brain tumor. Median survival is in the range of 12-15 months. The current standard of care includes surgery, radiotherapy and chemotherapy using concomitant and maintenance temozolomide (TMZ/RT→TMZ) (1). Accordingly, glioblastoma remains a disease area of high unmet clinical need.

Glioblastomas commonly (40%) exhibit amplification of the epidermal growth factor receptor (EGFR) gene. Half of these tumors carry a deletion mutation of exons 2-7 referred to as delta-EGFR or EGFRvIII which results in constitutive pathway activity. Multiple oncogenic properties of glioblastoma have been attributed to strong EGFR signalling, including migration, invasiveness, and resistance to apoptosis. Various therapeutic agents targeting amplified EGFR or EGFRvIII are currently being developed. Tyrosine kinase inhibitors (TKI) or monoclonal antibodies have failed to show benefit in clinical studies, typically in glioblastoma patient populations unselected for molecular marker profiles (2).

Antibody drug conjugates (ADC) have recently emerged as new promising therapeutic agents in oncology. They are composed of monoclonal antibodies to cell surface antigens which are connected to a cytotoxic agent, referred to as the toxic payload, via chemical linkers. Challenges of developing ADC are finding (i) the ideal antigens with high expression across the tumor, but with absent or low normal tissue expression, (ii) the ideal linker which prevents the release of the cytotoxic agent before targeting the antigen in order to prevent off-target effects, and (iii) potent on-target cytotoxic activity (3-5). Depatuxizumab mafodotin (ABT-414) (6) is an EGFR-targeting antibody drug conjugate consisting of the monoclonal antibody 806 (7) linked through a non-cleavable linker to a toxic payload, monomethyl auristatin F (MMAF) (8). The

extracellular domain of EGFR is composed of two cysteine-poor homologous large domains and two cysteine-rich domains. The epitope recognized by ABT-806 is located on the first cysteine-rich domain of the EGFR extracellular domain. This epitope is masked in the inactive tethered monomer state (tethered) or in the active ligand-bound "back-to-back" dimer state. Epitope exposure may occur as a result of extracellular domain truncation, as in EGFRvIII, or with overexpression of EGFR (6), (7).

MMAF binds to tubulins, disrupts microtubule dynamics and subsequently induces G2/M arrest and cell death (9-13). ABT-414 exhibits potent cytotoxic activity against glioblastoma patient-derived xenograft models expressing high levels of either wildtype EGFR or EGFRvIII (6). After early clinical trials in patients with recurrent in newly glioblastoma established ABT-414 safety and showed signs of drug activity (14), (15),(16), randomized clinicial trials have been conducted in recurrent (EORTC 1410, NCT02343406) and newly diagnosed (RTOG 3508, NCT02573324) glioblastoma.

ABT-806 targets a unique tumor-specific epitope of EGFR which is accessible when wildtype *EGFR* is amplified and always when EGFRvIII is expressed. The minimal reactivity with EGFR expressed in normal tissue made ABT-806 suitable to generate an ADC (7). ABT-414 bound to EGFR is internalized and processed within endosomes and lysosomes to release its lethal cargo. Yet, several questions regarding the precise mode of action of ABT-414 and potential pathways of constitutive or acquired resistance to ABT-414 among glioma cells remain unanswered. Here, we addressed some of them.

Materials and Methods

Reagents

Depatuxizumab mafodotin (ABT-414), mAb 806 and Ab095–vcMMAF (control ADC) were provided by AbbVie Inc. (Chicago, IL). The sequence of Depatuxizumab mafodotin is publicly available through the WHO INN publication online (https://www.who.int/medicines/publications/druginformation/innlists/RL77.pdf?ua=1). AB095 is an anti-tetanus toxin IgG1, which does not recognize any epitopes in mice and is therefore an appropriate isotype control. Gefitinib was obtained from InvivoGen (tlrl-gef; San Diego, CA), EGF was from PeproTech (Rocky Hill, NJ) and erlotinib from Selleckchem (Houston, TX). Details on cell lines and transfections are provided in Supplementary Note S1.

RT-PCR

RT-PCR was performed using the 2^(- delta CT) method (17). cDNA was produced by reverse transcription from total mRNA, applying the "High Capacity cDNA Reverse Transcription Kit" (Applied Biosystems by Thermo Fisher Scientific, Waltham, MA) and specific target gene expression, normalized to hypoxanthine-guanine phosphoribosyltransferase 1 (*HPRT1*), was determined using PowerUpTM SYBR Green Master Mix A25741 (Applied Biosystems by Thermo Fisher Scientific) and measured by the QuantStudio™ 6 real-time PCR system and QuantStudio software V1.2 (Thermo Fisher Scientific). Primer sequences were *EGFR* (forward 5'-GAGTCGGGCTCTGGAGGAAA-3', reverse 5'-CAGTTATTGAACATCCTCTGG AC-3') (18,19); *EGFRvIII* (forward 5'- GGCTCT GGAGGAAAAG AAAGGTAAT -3', reverse 5'-

CGACAG CTATGAGATGGAGGA -3') (20).

Immunoblot analysis

Immunoblot analysis and the nuclear/cytoplasmic sub-fractionation assay was performed as described (21). Antibodies were rabbit anti-phospho-EGFR (Tyr 1068) and anti-EGFR (1/1000, Cell Signaling Technology, CST, Danvers, MA), the signals of which were normalized to GAPDH (1/1000, EB07069, Everest Biotech Ltd, Bicester, UK) or to rabbit anti-lamin B1 (1/2000, Ab 16048, RRID:AB_443298, Abcam, Cambridge, UK) loading controls. HRP-conjugated secondary anti-rabbit antibody (1/5000, #7074, CST) was used as for detection.

Viability assays and flow cytometry

5'000-30'000 (5'000-15'000 for long-term glioma cell lines (LTC), 15'000-30'000 for glioma-initating cell (GIC) lines) for acute 72 h growth inhibition assays or 50-300 (50-100 for LTC, 100-300 for GIC) cells for clonogenicity or spherogenicity assays were seeded in their respective medium in 100 µl for LTC or 50 µL for GIC in a 96-well format 24 h prior to ABT-414 treatment at day 0. Cells were treated at day 1 either by replacement of the medium (100 µl + 1x drug concentration) for LTC or by adding medium (50 µl + 2x drug concentration) for GIC. The clonogenicity or spherogenicity assays were stopped when the first conditions reached approximately 80-90% confluency for adherent LTC cultures respectively 80% coverage of the culture dish area by spheres for GIC. Metabolic activity was assessed by 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) reduction either after 72 h for acute effects or after at least 10 days as a surrogate for colony formation (22). Senescence staining was performed using the Senescence β-galactosidase Staining Kit (Cell Signalling

Technology) (23). Details on flow cytometry are provided in Supplementary Note S2.

Animal studies

All animal experiments were conducted using standard operating procedures under a valid licence (ZH236/14) granted by the Cantonal Veterinary Office Zurich and Federal Food Safety and Veterinary Office. Immunocompromised CD1 nude mice (Charles River Laboratories, Sulzfeld, Germany) were xenografted with 75'000 LN-229 EGFRvIII cells. Stereotactic intracranial implantation was performed as previously described (23). Ten days post surgery mice were treated every 4 days for a total of six doses by intraperitoneal administration with ABT-414 or Ab095–vcMMAF (isotype control ADC) (10 mg/kg). Mice were assessed for neurological symptoms daily according to the Cantonal Veterinary Office Zurich guidelines. Three animals per treatment group and four animals per control groups were pre-randomized and euthanized when the first mouse became symptomatic. For survival analysis seven to eight animals per group were euthanized when developing neurological symptoms.

Histology and immunohistochemistry

Brains sections (8 µm) were fixed with paraformaldehyde, pretreated with 3% H_2O_2 and blocked with blocking solution (Biosystems, Muttenz, Switzerland). Sections were immunostained with a rabbit polyclonal antiserum (lot #111611, Celldex) that binds the EGFRvIII protein (antibody dilution: 1:2000). SignalStain® Boost IHC Detection Reagent (HRP, Rabbit) (#8114) (CST) and the ImmPact DAB kit (#SK-4105, Vector Laboratories, Burlingame, CA) were used to visualize immune reactions.

Statistical analysis

Representative data of experiments, performed in technical and usually three biological replicates, are shown here and are presented as mean values and standard deviations. One-way as well as two-way ANOVA with Bonferroni post-hoc testing (multiple comparisons) were used for statistical analyses. Antagonistic effects were calculated by the fractional product method (24) and differences of >10% of the observed versus predicted ($\frac{treatment\ A \times treatment\ B}{100}$) reduction of viability were considered as indicative of synergy or antagonism.

Results

Generation and characterization of EGFR↑ and EGFRvIII glioma models

We have recently characterized a panel of human LTC and GIC for the expression and activity of EGFR and their sensitivity to the EGFR tyrosine kinase inhibitor, gefinitib (21). These cell lines were uniformly refractory to ABT-414 at concentrations of up to 10 nM for exposure times of up to 72 h (Fig. S1A). Therefore, we obtained stable transfected sub-cell lines of two LTC (U87MG, LN-229) and one GIC (S-24) overexpressing wildtype EGFR, referred to herein as EGFR↑ or EGFRvIII (Fig. 1A). Immunoblot demonstrated that overexpression of EGFR or EGFRvIII resulted in enhanced EGFR or EGFRvIII phosphorylation (Fig. 1B). Cell surface staining for wildtype EGFR or EGFRvIII resulted in increased specific fluorescence indexes (SFI) for EGFR↑ cells using an anti-EGFR wildtype specific antibody and for EGFRvIII-expressing cells using an anti-EGFRvIII specific antibody (Fig. 1C). Exposure to exogenous EGF increased pEGFR levels strongly in EGFR↑ cells, more so in U87MG and LN-229 than in S-24 cells. EGFRvIII is known not to bind EGF. Yet, in EGFRvIII-transfected LN-229 and S-24 cells, EGF induced pEGFR to higher levels than in control-transfected cells, indicating that the co-expression of EGFRvIII facilitates phosphorylation of EGFR in response to EGF (Fig. 1D, Fig. S2A). Removal of EGF from the culture medium for 48 h decreased pEGFR remarkably in EGFR↑ cells, but also to a lesser extent in EGFRvIII cells, further substantiating a role of endogenous EGFR mediating EGFRvIII phosphorylation in response to EGF (Fig. 1E, Fig. S2B). EGFRvIII cells showed a significant growth advantage over control or EGFR↑ cells for all three models. U87MG EGFR↑ cell proliferation was enhanced over control, too (Fig. 1F). Since EGF is an essential component of the medium used to maintain GIC cultures, we next explored whether ectopic EGFR or EGFRvIII

expression rendered the cells less dependent on EGF for survival and sphere formation. Indeed, removal of EGF from the culture medium resulted in growth inhibition of S-24 control and EGFR↑ cells whereas S-24 EGFRvIII cells required no external EGF for growth (Fig. S3A,B).

Ectopic EGFR expression confers glioma cell sensitivity to ABT-414 in vitro

EGFR phosphorylation was largely refractory to ABT-414 exposure at 24 h in EGFR↑ cells whereas phosphorylation was reduced by ABT-414 in all EGFRvIII cell lines. Total EGFR was unaffected in EGFR↑ cells; total transfected EGFRvIII was unaffected in U87MG and S-24, but moderately reduced in LN-229 cells (Fig. 2A, Fig. S2C). In 72 h exposure assays, expression of EGFRvIII induced sensitivity to ABT-414 in all three models examined, U87MG, LN-229 and S-24, whereas overexpression of wildtype EGFR induced moderate sensitization in U87MG and LN-229 only; Of note, S-24 may be less sensitive to ABT-414 in these short-term assays because of slower proliferation rate compared to U87MG or LN-229 (Fig. 2B). There was thus correlation between early effects on pEGFR levels and effects on growth 48 h later. The specificity of killing was verified by the demonstration of rescue of cell death by unarmed ABT-806 antibody (Fig. S1B). Next, we investigated the effect of ABT-414 treatment on clonogenicity (LTC) or spherogenicity (GIC). Growth of EGFRvIII cells was again strongly inhibited even by low concentrations of ABT-414. Overexpression of wildtype EGFR induced sensitivity to ABT-414 in these assays, too, in all cell lines (Fig. 2C, Table S1). The cytotoxic agent MMAF is known to inhibit the polymerization of tubulin as well as destabilizing microtubule structures once inside the cell causing cell cycle arrest in G2/M (25). Since we observed that, in the duration of the assay, ABT-414 prevented proliferation to a greater extent than causing acute cytotoxicity, in particular in EGFR↑ cells, we performed cell cycle analysis. Treatment with ABT-414 for 72 h induced a minor

G2/M arrest in EGFRvIII cells with a fold difference of 1.8 for LN-229 and S-24 cells, but no such effect in EGFR↑ LN-229 cells and S-24 cells. There was also relative increase in sub-G1, which represents the dead or dying cell fraction, in both EGFR↑ and EGFRvIII cells (Fig. 2D). Altogether, these data suggest that cell death induced by ABT-414 is delayed and underestimated when assessed at 72 h (Fig. 2B,C). β-galactosidase staining indicated that induced senescence may mediate some of the delayed effects of ABT-414 in LN-229 EGFR↑ and EGFRvIII sub-cell lines (Fig. 2E), although no such effects were seen in S-24 (Fig. S4).

ABT-414 prolongs survival in an EGFRvIII overexpressing orthotopic mouse glioma model

The efficacy of ABT-414 *in vivo* was assessed in the three LN-229 human orthotopic xenograft models in nude mice. A comparison of PBS-treated controls showed that the expression of wild-type EGFR and, even more prominently, of EGFRvIII in tumors decreased animal survival (Fig. 3). There was no difference between Ab095-vcMMAF and PBS treatment in either model, whereas ABT-414 specifically and profoundly prolonged survival of mice carrying EGFRvIII-expressing tumors (Mantel-Cox test). ABT-414 did not prolong survival in mice carrying tumors generated by control-transfected cells or wild-type EGFR-overexpressing cells however (Figure 3A,C,E). Immunohistochemistry for EGFRvIII confirmed EGFRvIII expression in EGFRvIII-transfected cells, and demonstrated loss of expression in tumors from mice treated with ABT-414 but not with control PBS or Ab095-vcMMAF (Fig. 3F, Fig. S5). When tumors from mice treated with Ab095-vcMMAF or ABT-414 were harvested from end-stage animals several weeks after conclusion of therapy, loss of EGFRvIII had persisted in ABT-414-treated animals (Fig. S6A). Furthermore, EGFRvIII expression was not regained even after 4 weeks passaging in cell culture (Fig. S6B).

Synergy of ABT-414 with irradiation or alkylating agents in vitro

With a view on the clinical application of ABT-414, we also asked whether ABT-414 activity was negatively or positively affected by hypoxia, a typical feature of glioblastoma, or co-exposure to irradiation or two alkylating agents commonly used in this disease setting, TMZ and lomustine. The sensitivity of EGFR↑ cells to ABT-414 was essentially unaltered under hypoxia whereas EGFRvIII cells were moderately more resistant under hypoxic conditions (Fig. S7). Then we tested whether lomustine, TMZ or irradiation may induce upregulation of EGFR expression and its activation, which might enhance ABT-414 binding activity. However, only minor pEGFR induction was observed. Longer exposure revealed either similar results or no alteration of pEGFR levels at all (Fig. S8A,C). Co-exposure to ABT-414 and lomustine, TMZ or irradiation resulted in largely additive, rarely synergistic, but never antagonistic effects (Fig. S8B,D).

No bystander effect of ABT-414

Next, we explored whether cytotoxic effects of ABT-414 are strictly limited to cells overexpressing EGFR, or whether there could also be some level of bystander killing, e.g., from free toxin circulating from cell to cell.

We co-cultured EGFRvIII LN-229 or ZH-161 cells with LN-229 or ZH-161 cells expressing GFP only and treated these mixed cultures with ABT-414. In these studies, cell death induction was restricted to non-GFP-expressing cells, excluding any relevant bystander effect *in vitro* (Fig. 4).

Ligand stimulation limits rather than promotes ABT-414 activity

Since ABT-414 is supposed to recognize a specific activation-related conformation of EGFR (6), we also explored whether co-exposure to EGF concentrations sufficient to induce pEGFR sensitized

cells to ABT-414 by promoting the "active" EGFR conformation. This was not the case (Fig. S9A). Flow cytometry demonstrated that pre-stimulation of cells with EGF for 15 min rather decreased cellular reactivity with mAb 806, the antibody moiety of ABT-414, more so in LN-229 than in S-24 cells (Fig. S9B,C). In agreement, pre- or post-stimulation with EGF relative to ABT-414 treatment conferred minor antagonism particularly in U87MG control and EGFR↑ cells, in LN-229 EGFR↑ and EGFRvIII cells and in S-24 EGFRvIII cells, and EGF stimulation alone increased proliferation in some models (Fig. S9D,E). The antagonistic effects of EGF in the EGFRvIII models once more suggest a role for endogenous EGFR in regulating overall signaling activity. In summary, no synergy was observed by combining EGF-induced EGFR phosphorylation and ABT-414 treatment. On the contrary, slight antagonism was detected, suggestive of EGF-mediated receptor internalization, leading to lower EGFR density at the cell surface and relative resistance to ABT-414, as well as enhanced proliferation.

EGFR TKI limit rather than promote ABT-414 activity

Given these data, we wondered whether inhibiting rather than activating the EGFR pathway might increase EGFR availability for ABT-414 binding at the cell surface. However, when the glioma cells were exposed to gefitinib at concentrations sufficient to inhibit EGFR phosphorylation (21), there was strong reduction of mAb 806 binding, suggesting that EGFR dephosphorylation decreases ability of ABT-414 binding (Fig. 5A). However, flow cytometry using an EGFR antibody that recognizes both EGFR wildtype and EGFRvIII, revealed decreased labeling too (Fig. 5B), suggesting loss of EGFR protein from the cell surface upon gefitinib treatment. Gefitinib treatment induced decreased pEGFR levels as well, and led to a strong decrease of total EGFR wildtype and EGFRvIII protein as detected by immunoblots of total cellular lysates (Fig. 5C). This effect was not

specific to gefitinib since erlotinib had similar effects in S-24 cells and, was not restricted to glioma cells since A431 cells showed the same response to erlotinib (Fig. S10A,B).

Next, we asked whether the combination of ABT-414 with gefitinib would also result in antagonism, given that gefitinib at certain concentrations reduces expression of the target of ABT-414. Indeed, co-exposure to ABT-414 and gefitinib revealed antagonism in LN-229 EGFR↑ and EGFRvIII, as well as in S-24 EGFRvIII cells (Fig. 5D).

EGFR phosphorylation is dispensable for ABT-414 activity

To further test the premise of decreased mAb-806 binding activity upon receptor deactivation, experiments using kinase-dead EGFR↑ and EGFRvIII cells (26) were performed. EGFR phosphorylation was reduced or lost in kinase-dead EGFR↑ or EGFRvIII cells, and stimulation with EGF only moderately induced phosphorylation compared to the strong effect in kinase-active cells (Fig. 6A, Fig.S2D). In order to further explore the loss of EGFR protein and the relevance of EGFR phosphorylation for its effective targeting, we performed flow cytometry and immunoblot with an antibody targeting wildtype EGFR and EGFRvIII. Stimulation with EGF decreased antibody binding in EGFR↑ and EGFRvIII, but not in kinase-dead cells. Furthermore, phosphorylation increased upon EGF stimulation in kinase-active, but not in kinase-dead cells. However, both in kinase-active as well as in kinase-dead cells, treatment with gefitinib more than with ABT-414 decreased EGFR on the cell surface, measured by flow cytometry and immunoblot (Fig. 6B,C). Finally, sensitivity to ABT-414 was invariable in both kinase-dead cells as well as in kinase-active cells (Fig. 6D).

Discussion

ABT-414, a novel ADC targeting EGFR, has shown promising clinical activity in patients with EGFR-amplified recurrent glioblastoma (15,27,28). However, among these patients, it has remained challenging to identify which patients respond and which do not, and a significant fraction of patients appears not to respond, despite high level EGFR amplification.

Since ABT-414-mediated cell killing correlates with EGFR density at the cell surface *in vitro* *(6)*, we first sought to correlate EGFR and EGFRvIII expression with the cytotoxic activity of ABT-414 in 9 LTC and 5 GIC glioma models. The uniform resistance of these glioma cell lines to ABT-414 was somewhat surprising since some of these cell lines exhibit robust EGFR expression (Fig. S1A) (21). Phillips *et al.* (2016) reported that ABT-414 displayed cytotoxic effects in A431, an EGFR wildtype amplified vulvar epidermoid carcinoma cell line and in HCC827.ER.LMC, an EGFR wildtype amplified lung adenocarcinoma cell line only, with EC_{50} values $\leq$ 10 nM. Concentration response studies in our study revealed that 10 nM was the highest applicable, non-toxic dose for the MMAF control ADC. The EC_{50} values of other EGFR wildtype amplified cell lines tested by Phillips *et al.* (2016) were above 10 nM. Constitutive cancer cell line sensitivity to ABT-414 *in vitro* thus seems to be rare.

EGFR amplification and expression of EGFRvIII mutation are lost *in vitro* upon culturing of primary glioma cells (29,30). Thus, we generated stable transfected sub-cell lines, designed to either overexpress EGFR wildtype (EGFR↑) or EGFRvIII (Fig. 1). In our model systems, early loss of phosphorylated EGFRvIII correlated best with the activity of ABT-414 (Fig. 2A, Fig. S2C). Overexpression of EGFRvIII in cell lines resulted in robust ABT-414-mediated cytotoxicity whereas overexpression of wildtype EGFR had a lesser effect (Fig. 2B). ABT-414 had more

prominent effects in clonogenicity than in acute cytotoxicity assays with EC_{50} values ranging from 2.1-8.43 nM versus > 10 nM in EGFR↑ cells and 7.8 x 10^{-5}-0.03 nM versus 0.41-6 nM in EGFRvIII cells (Fig. 2B,C, Table S1). Cell cycle analyses after 72 hours exposure did not reveal specific changes induced by ABT-414, suggesting that G2/M arrest-independent, delayed cell death is likely the best explanation for more prominent effect of ABT-414 with prolonged exposure. In addition, the toxic payload may induce features of senescence in some cell lines (Fig. 2E). Strong anti-glioma activity of ABT-414 was confirmed *in vivo* in the orthotopic LN-229 EGFRvIII glioma xenograft model. EGFRvIII expression was virtually lost in tumors of mice that had been treated with ABT-414, likely as a consequence of selection of cells with little or no transcription from the plasmid, demonstrating the long-lasting effect of ABT-414 *in vivo* as long as the target is expressed, but also highlighting potential escape pathways from ABT-414 treatment (Fig. 3, S5, S6).

The EORTC 1410 trial, which evaluated the efficacy of ABT-414 plus TMZ versus ABT-414 alone versus TMZ or lomustine (CCNU) alone (NCT02343406) suggested a trend toward a better activity for the combination of ABT-414 and TMZ, although the results were not statistically significant ($p>0.05$) (31). Combination experiments *in vitro* with ABT-414 and with lomustine, TMZ or irradiation showed predominantly additive effects in our model systems *in vitro* (Fig. S8).

The activity of ABT-414 is believed to result from internalization of EGFR bound to ABT-414 and subsequent degradation in lysosomal compartments, leading to release of Cys-mc-MMAF (32,33). Furthermore, it is assumed that MMAF exhibits no cell membrane permeability which prevents bystander killing (34,35). Co-culture studies indeed confirmed the absence of bystander killing in our experimental set-ups (Fig. 4).

Based on the suggested binding properties of ABT-414 (11,12,36), we anticipated that pre- or co-exposure to exogenous EGF would enhance the binding activity of ABT-414, due to the

suggested stabilization of the EGFR extended conformation by the ligand (36). This was not the case. In contrast, EGF stimulation of EGFR wildtype- or vIII-overexpressing cells rather decreased the reactivity with mAb 806 (Fig. S9A-E). Ligand-induced EGFR dimerization or oligomerization eventually enhances internalization (37,38), which would prevent mAb 806 binding. In line with this, pre-exposure of cells to EGF interfered with the activity of ABT-414 (Fig. S9F,G). Similar results were observed in BaF/3, a murine interleukin-3 dependent pro B cell line. Pre-incubation with EGF decreased the reactivity with mAb 806, too; however, the authors claimed that this effect was not due to internalization of the receptor but rather through steric hindrance or masking of the epitope by the ligand (36).

MAb 806 is believed to bind to the extended, untethered conformation (active) of EGFR which occurs as EGFR moves from a tethered conformation (inactive) to an active "back-to-back" dimer state (11). Preventing activation of EGFR using the tyrosine kinase inhibitor, gefitinib, decreased mAb 806 activity, but, surprisingly, more likely due to loss of EGFR protein from the cell surface upon gefitinib treatment than due to a conformational change of EGFR (Fig. 5A-D).

Furthermore, using LN-229 sub-cell lines expressing kinase-dead EGFR or EGFRvIII, we demonstrate that phosphorylation of EGFR is dispensable for binding of mAb 806 as well as for the cytotoxic activity of ABT-414 or gefitinib (Fig. 6A-D). It was previously shown that mutations in the tyrosine kinase domain do not affect the binding affinity of gefitinib in non-small-cell lung cancer cells (39). However, the preserved activity of ABT-414 in the kinase-dead models represent potentially the most important novel finding reported here because these data, albeit in an artificial model, dissociate EGFR phosphorylation from the "active" conformation thought to be required for the binding of mAb 806.

Accordingly, expression of EGFR at the cell surface rather than EGFR phosphorylation may be the best predictor of benefit from ABT-414 at the level of the target. Efforts to modulate sensitivity of tumors to ABT-414 by modulating EGFR levels, through either ligand-dependent stimulation or, more importantly, pharmacological inhibition using tyrosine kinase inhibitors, should therefore be explored with caution and may actually be counterproductive.

Our study has limitations. Data were obtained with artificial models engineered to overexpress wildtype EGFR or EGFRvIII because there was no other option to elucidate modes of action of ABT-414. We did not explore the kinetics of EGFR internalization with or without EGF or EGFR inhibitors nor did we explore the down-stream mechanisms of EGFR regulation such as ubiquitination, although an increasing body of literature suggests a role for ligand-independent trafficking of EGFR (40,41). Although EGFRvIII rather than EGFR overexpression uniformly induced glioma cell sensitivity to ABT-414 (Table S1), EGFRvIII expression has not emerged as a biomarker for benefit from ABT-414 in the clinic (31), potentially challenging the clinical relevance of our observations. A simple explanation for the higher sensitivity of EGFRvIII cells *in vitro* would be their increased growth rate compared with EGFR↑ cells. Pre- and post-ABT-414 exposure studies in human patients will clarify whether EGFRvIII-expressing are still preferentially eliminated. Since all EGFRvIII-positive tumors overexpress EGFR wildtype too, preferential elimination of the fraction of EGFRvIII-expressing cells, which is highly variable in human glioblastoma, may not affect overall outcome in patients.

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Figure Legends

Figure 1. Generation and characterization of glioma cells expressing wildtype EGFR or EGFRvIII. A,B. Characterization of EGFR or EGFRvIII mRNA expression by RT-PCR (A, y-axis representing mRNA of EGFR or EGFRvIII relative to HPRT1) or protein levels by immunoblot (wildtype (wt) EGFR: 175 kDa, EGFRvIII: ~140 kDa) (B). C. Cell surface EGFR was assessed by flow cytometry with anti-EGFR-FITC recognizing EGFR wildtype only (upper rows) or anti-EGFRvIII-PE recognizing specifically EGFRvIII (lower rows). Specific fluorescence indexes (SFI) were calculated by dividing the mean fluorescence intensity of anti-EGFR-FITC- or anti-EGFRvIII-PE-labelled cells by the mean fluorescence intensity of isotype control-labelled cells and are included in the right upper corners of each panel. D,E. EGFR phosphorylation without and with EGF exposure (50 ng/ml, 15 min) (D) or with and without EGF deprivation for 48 h (E) determined by immunoblot. F. Limiting dilution clonogenicity (LTC) or spherogenicity (GIC) of control (ctr), EGFR↑ or EGFRvIII cells. GAPDH was used as a loading control for immunoblot. Metabolic activity was assessed by MTT assay after 10-20 days. Data are expressed as mean ± SEM (*$p<0.05$, ***$p<0.001$: EGFR↑ or EGFRvIII versus ctr, two-way ANOVA with Bonferroni correction).

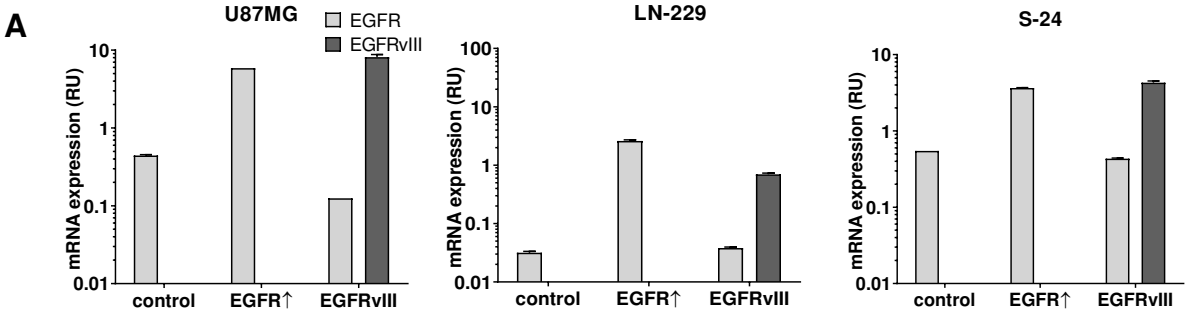
Figure 2. Ectopic EGFR or EGFRvIII expression induce sensitivity to ABT-414. A. Cells were exposed to ABT-414 or Ab095–vcMMAF control toxin (10 nM, 24 h) and whole cell lysates were assessed for phosphorylated and total EGFR by immunoblot (wildtype EGFR: 175 kDa, EGFRvIII: ~140 kDa), using GAPDH as a loading control. B,C. LN-229, U87MG or S-24 control-transfected (ctr), EGFR↑ or EGFRvIII cells were exposed to ABT-414 or Ab095–vcMMAF control toxin. Metabolic activity after 72 h (B) or clonogenic survival (C) were assessed by MTT assay (means and SEM, n=3, *$p<0.05$, ***$p<0.001$: ABT-414 versus control toxin, two-way ANOVA with

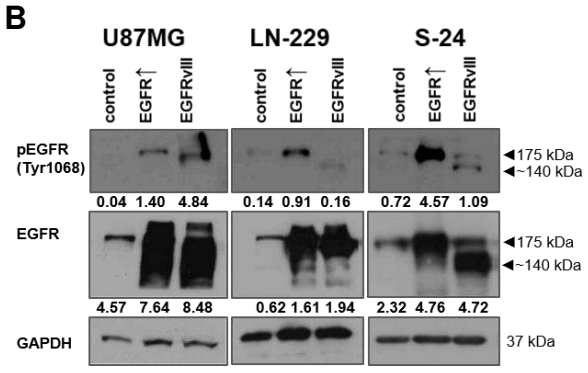
582 Bonferroni correction). D. LN-229 or S-24 sublines were treated with 10 nM ABT-414 or Ab095–
583 vcMMAF control toxin for 72 h and cell cycle distribution was measured by PI staining. E. LN-229
584 sub-cell lines were treated with ABT-414 or control antibody for 72 h and subsequently subjected to
585 β-galactosidase staining, using irradiation at 20 Gy as a positive control.
586
587 **Figure 3. ABT-414 prolongs survival in the orthotopic EGFRvIII LN-229 glioma model**. Mice
588 were inoculated with LN-229 control (A,B), EGFR↑ (C,D) or EGFRvIII (E,F) cells and treated
589 every 4 days for a total of six doses with PBS, Ab095–vcMMAF or ABT-414 (8 mg/kg). Survival is
590 shown by Kaplan-Meier curves (median survival in days provided in brackets) (A,C,E).
591 Prerandomized animals were euthanized at day 32 and cryosections of the brains were stained with
592 a polyclonal rabbit anti-EGFRvIII antibody. Representative stainings of mice treated with Ab095–
593 vcMMAF or ABT-414 are depicted (size bar: 100 µm).
594
595 **Figure 4. No bystander killing of EGFRvIII-negative glioma cells by ABT-414.** LN-229 (A, C)
596 or ZH-161 (B, D) EGFRvIII- or GFP-transfected cells were exposed to 10 nM ABT-414, either
597 each cell line alone in a well or both cell lines together in a well, in a co-culture assay for 72 h. Cell
598 killing was assessed by Zombie-NIR, a cell impermeable dye to label cells with compromised
599 membranes, and then measured by flow cytometry. Cell populations were divided into GFP-positive
600 cells, EGFRvIII cells, or both cell lines together and the alive or respective dead cells were
601 measured for the GFP-positive and the GFP-negative cell population, and the respective cell
602 fractions are given in percentages.
603

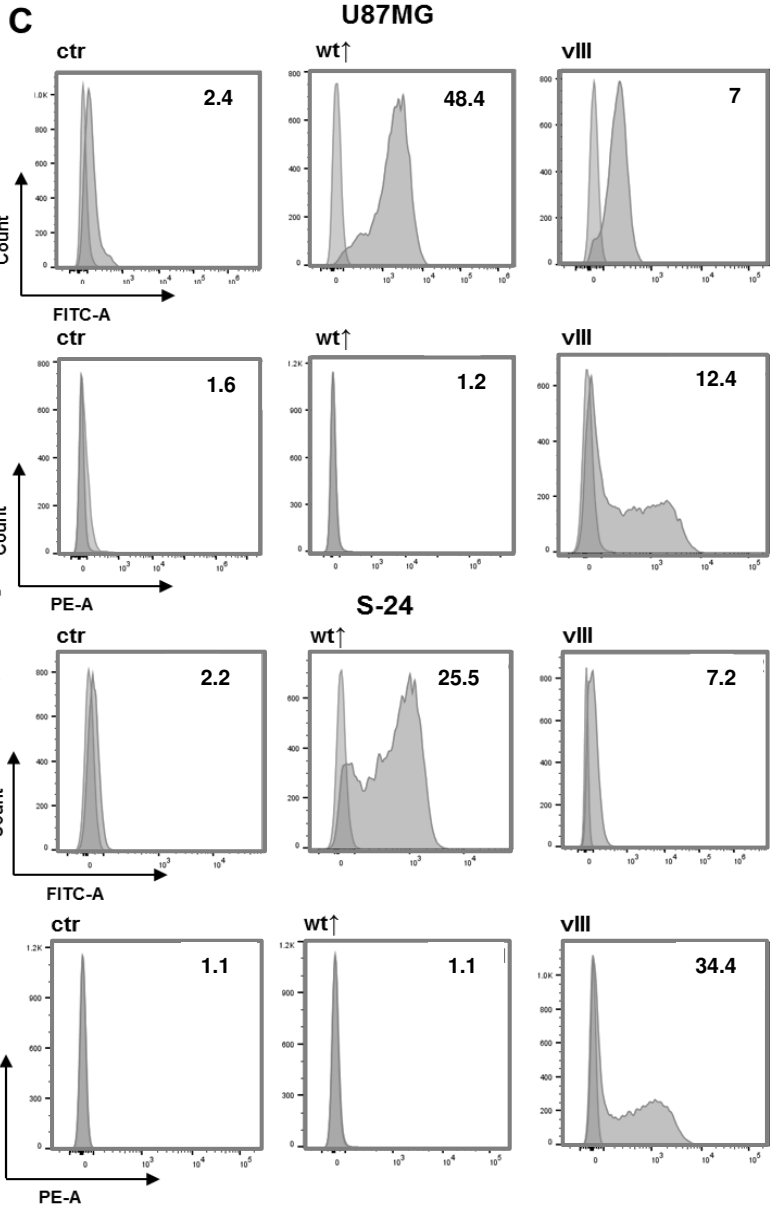
Figure 5. Effect of EGFR tyrosine kinase inhibition on mAb 806 binding activity. A,B. LN-229 cells were pretreated for 24 h with gefitinib and then labelled either with mAb-806-PE (A) or with anti-EGFR-FITC recognizing both wildtype EGFR and EGFRvIII (B). C. Phosphorylated and total EGFR of gefitinib-treated (24 h) LN-229 sub-cell lines were measured by immunoblot, using GAPDH as a loading control (left: short exposure, right: long exposure of the same blot). D. LN-229 or S-24 sub-cell lines were pretreated with gefitinib (5 μM) 4 h, then 10 nM ABT-414 for parental or EGFR↑ cells or 0.1 nM for U87MG and LN-229 EGFRvIII cells or 1 nM for S-24 EGFRvIII was added. Metabolic activity was measured after 72 h by MTT assay and is expressed normalized to vehicle control (* antagonistic effect defined as difference of >10% of observed versus predicted effect).

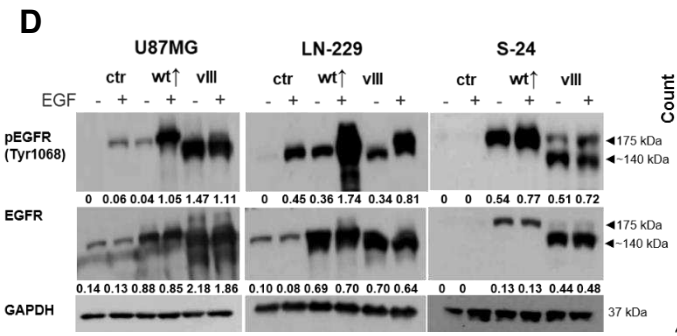
Figure 6. EGFR phosphorylation is not required for mAb 806 binding and ABT-414 cytotoxicity. A. EGFR↑ or EGFRvIII cell lines without or with the kinase-dead (D813N) mutation were examined for pEGFR and total EGFR levels by immunoblot. The cells were either pre-stimulated with EGF (50 ng/ml, 15 min) or not. B. The cell lines were either stimulated with EGF (50 ng/ml, 15 min) or treated with gefitinib (5 μM), using DMSO as control or ABT-414 (10 nM), using MMAF as control, and levels of EGFR at the cell surface were measured by flow cytometry with anti-EGFR-FITC recognizing both wildtype EGFR and EGFRvIII (B) or pEGFR and EGFR were measured in whole cell lysates by immunoblot (C). D. LN-229 EGFR↑ or EGFRvIII cell lines without or with the kinase-dead (D813N) mutation were exposed to ABT-414 or Ab095–vcMMAF control toxin and metabolic activity was measured after 72 h by MTT assay.

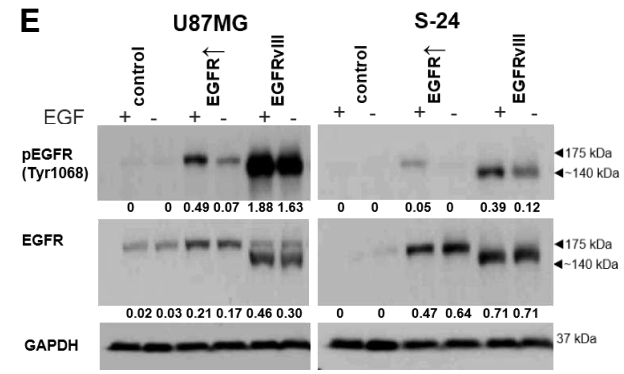
Figure 1


A
U87MG
LN-229
S-24
EGFR
EGFRvIII
mRNA expression (RU)
control
EGFR↑
EGFRvIII


B
U87MG
LN-229
S-24
control
EGFR↑
EGFRvIII
pEGFR (Tyr1068)
EGFR
GAPDH
175 kDa
~140 kDa
37 kDa
0.04 1.40 4.84
0.14 0.91 0.16
0.72 4.57 1.09
4.57 7.64 8.48
0.62 1.61 1.94
2.32 4.76 4.72


C
U87MG
ctr
wt↑
vIII
2.4
48.4
7
1.6
1.2
12.4
S-24
2.2
25.5
7.2
1.1
1.1
34.4
Count
FITC-A
PE-A


D
U87MG
LN-229
S-24
ctr
wt↑
vIII
EGF
pEGFR (Tyr1068)
EGFR
GAPDH
175 kDa
~140 kDa
37 kDa
0 0.06 0.04 1.05 1.47 1.11
0 0.45 0.36 1.74 0.34 0.81
0 0 0.54 0.77 0.51 0.72
0.14 0.13 0.88 0.85 2.18 1.86
0.10 0.08 0.69 0.70 0.70 0.64
0 0 0.13 0.13 0.44 0.48


E
U87MG
S-24
control
EGFR↑
EGFRvIII
EGF
pEGFR (Tyr1068)
EGFR
GAPDH
175 kDa
~140 kDa
37 kDa
0 0 0.49 0.07 1.88 1.63
0 0 0.05 0 0.39 0.12
0.02 0.03 0.21 0.17 0.46 0.30
0 0 0.47 0.64 0.71 0.71

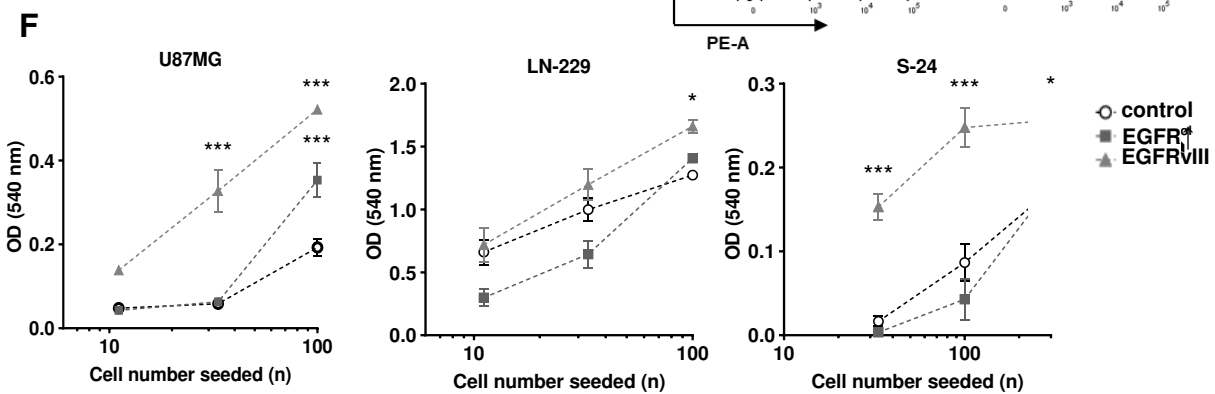
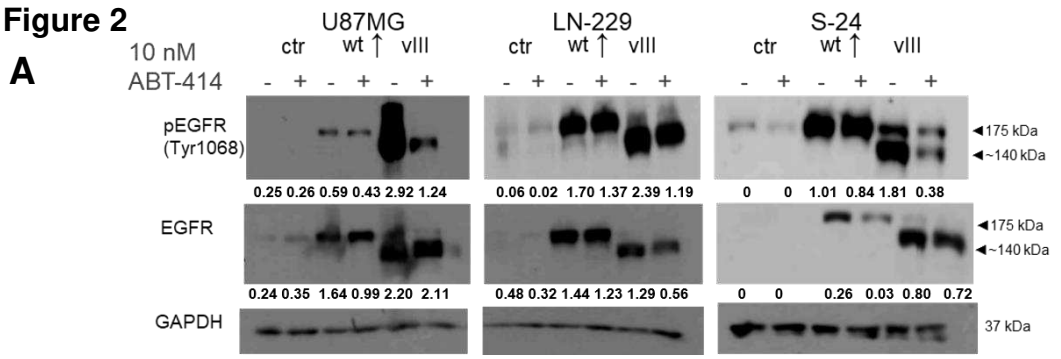
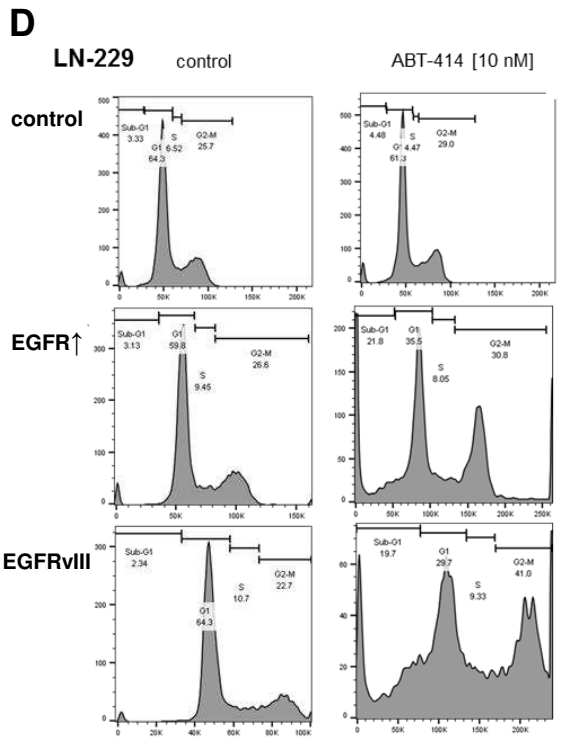
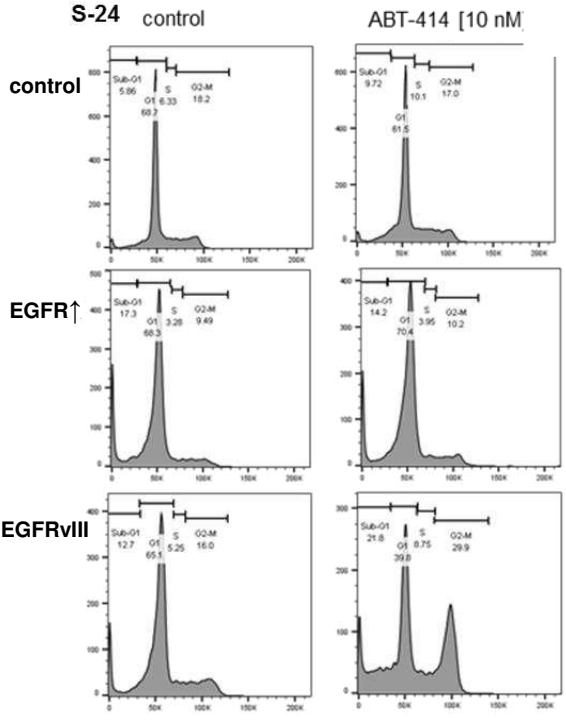
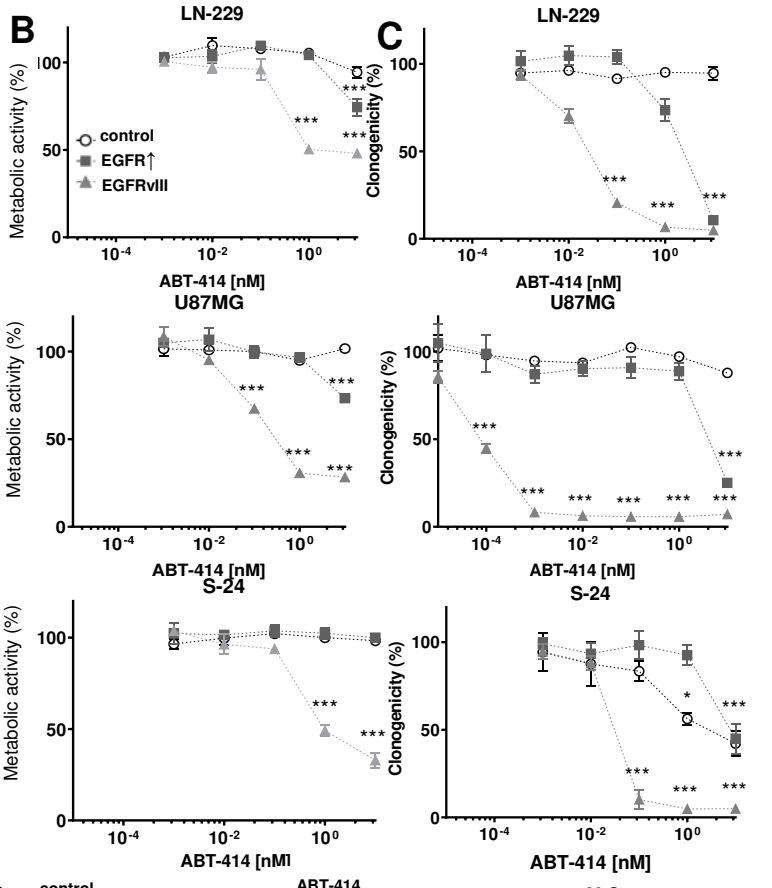

F
U87MG
LN-229
S-24
OD (540 nm)
Cell number seeded (n)
control
EGFR↑
EGFRvIII
***
*

Figure 2


A
10 nM ABT-414
U87MG
LN-229
S-24
ctr
wt ↑
vIII
pEGFR (Tyr1068)
EGFR
GAPDH
◄175 kDa
◄~140 kDa
37 kDa
0.25 0.26 0.59 0.43 2.92 1.24
0.06 0.02 1.70 1.37 2.39 1.19
0 0 1.01 0.84 1.81 0.38
0.24 0.35 1.64 0.99 2.20 2.11
0.48 0.32 1.44 1.23 1.29 0.56
0 0 0.26 0.03 0.80 0.72


D
LN-229
control
ABT-414 [10 nM]
control
EGFR↑
EGFRvIII


S-24
control
ABT-414 [10 nM]
control
EGFR↑
EGFRvIII


B
C
LN-229
U87MG
S-24
Metabolic activity (%)
Clonogenicity (%)
control
EGFR↑
EGFRvIII
ABT-414 [nM]

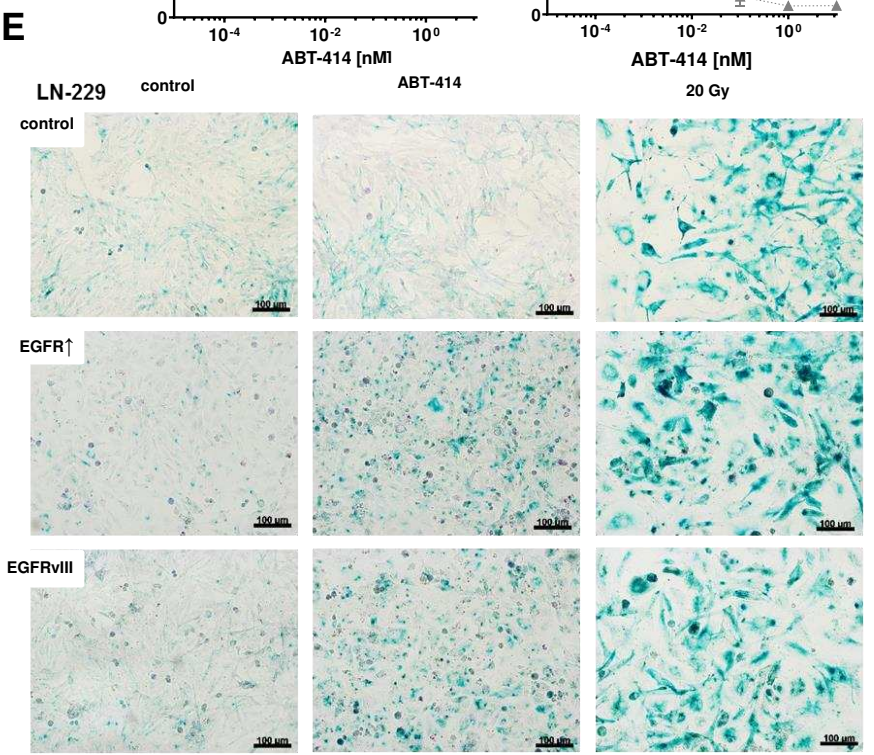

E
LN-229
control
ABT-414
20 Gy
control
EGFR↑
EGFRvIII

Figure 3

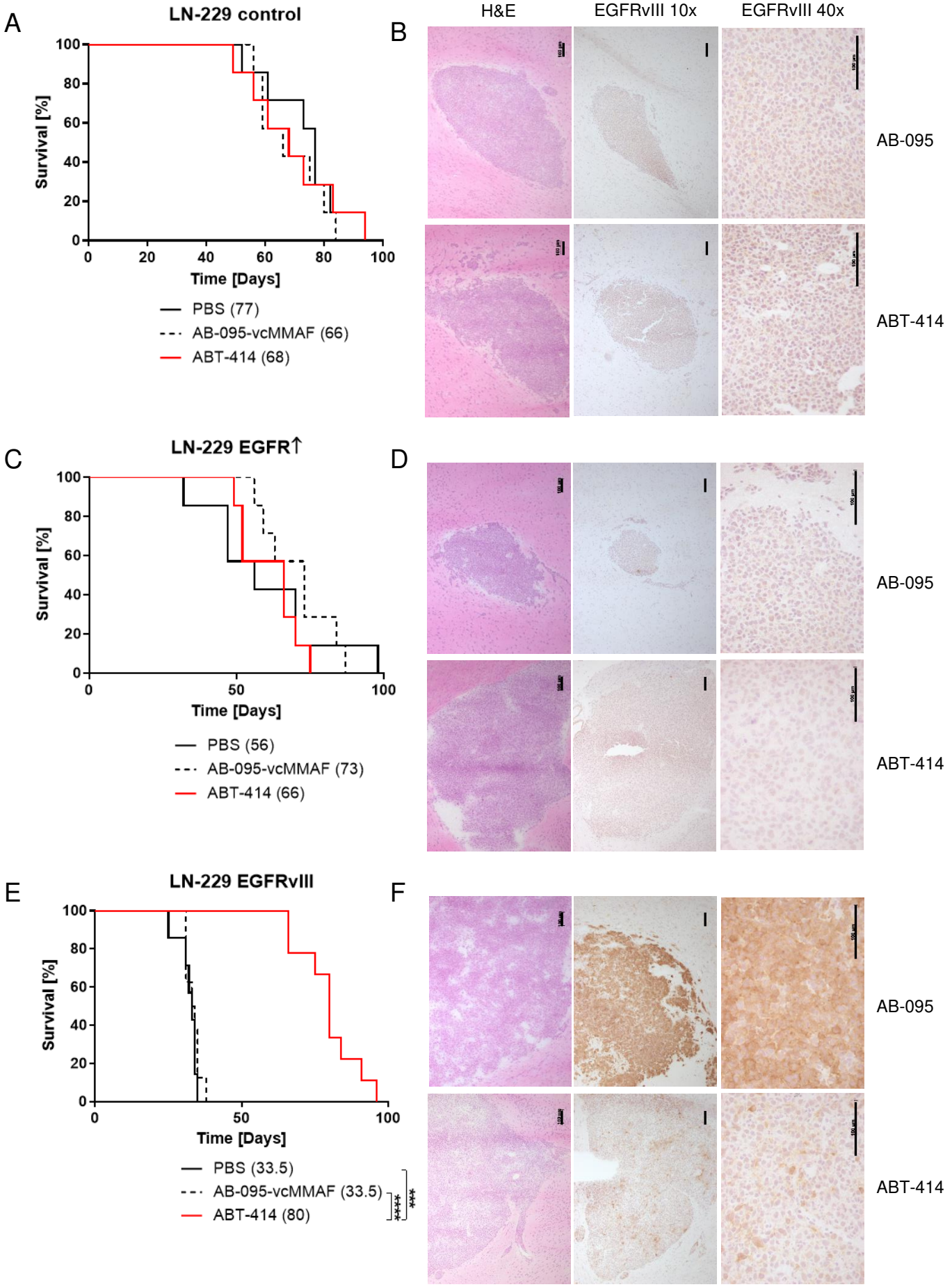
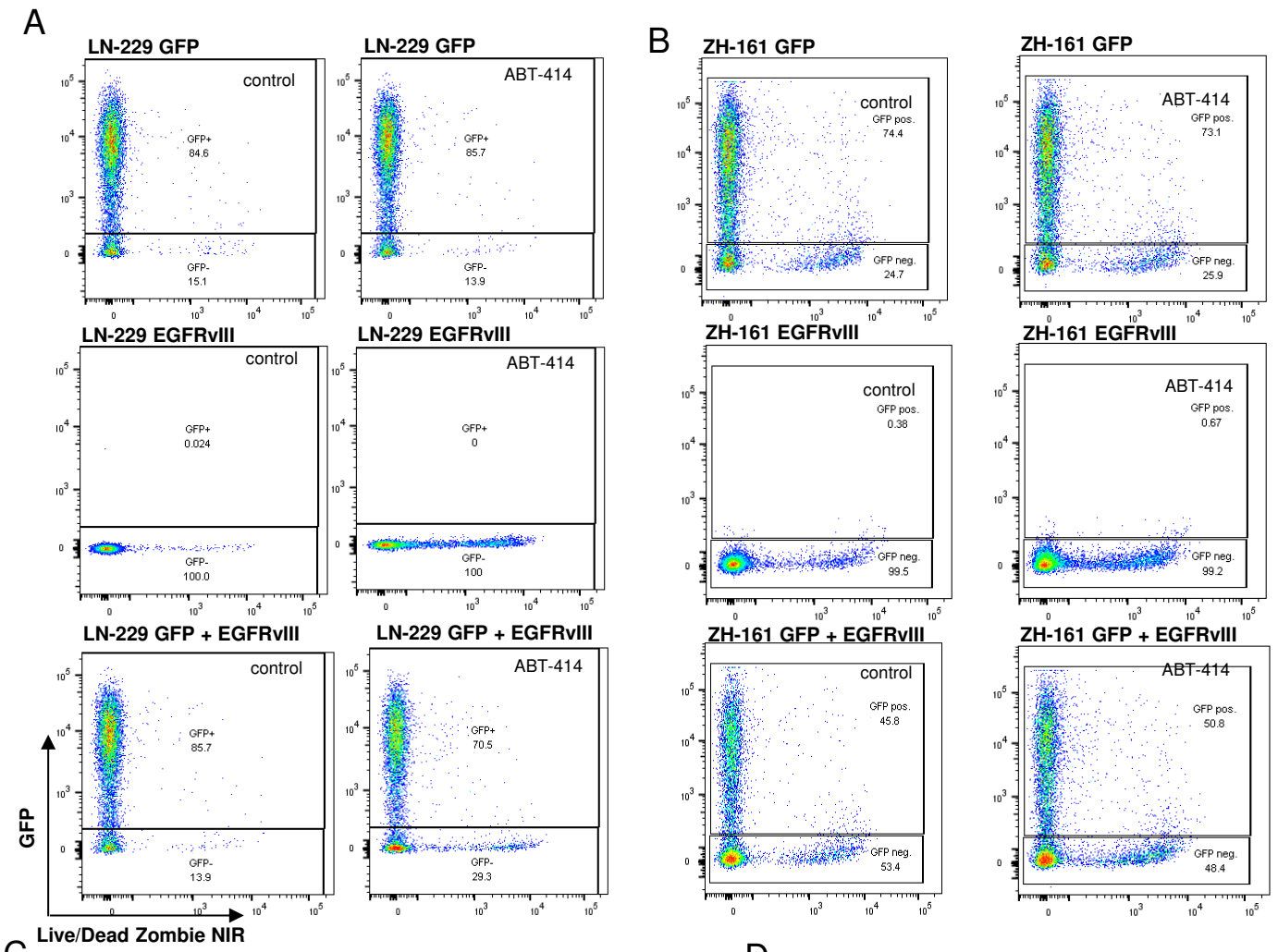
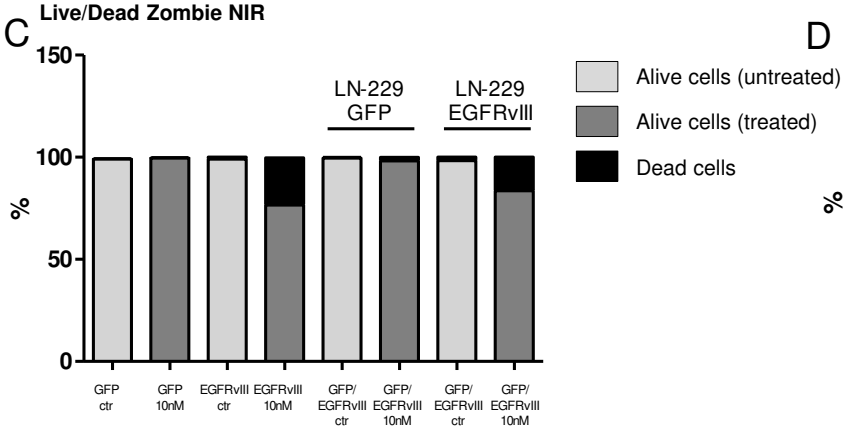

A
LN-229 control
Survival [%]
Time [Days]
PBS (77)
AB-095-vcMMAF (66)
ABT-414 (68)
B
H&E
EGFRvIII 10x
EGFRvIII 40x
AB-095
ABT-414
C
LN-229 EGFR↑
Survival [%]
Time [Days]
PBS (56)
AB-095-vcMMAF (73)
ABT-414 (66)
D
AB-095
ABT-414
E
LN-229 EGFRvIII
Survival [%]
Time [Days]
PBS (33.5)
AB-095-vcMMAF (33.5)
ABT-414 (80)
****
***
F
AB-095
ABT-414

Figure 4


A
LN-229 GFP
control
GFP+ 84.6
GFP- 15.1
LN-229 GFP
ABT-414
GFP+ 85.7
GFP- 13.9
LN-229 EGFRvIII
control
GFP+ 0.024
GFP- 100.0
LN-229 EGFRvIII
ABT-414
GFP+ 0
GFP- 100
LN-229 GFP + EGFRvIII
control
GFP+ 85.7
GFP- 13.9
LN-229 GFP + EGFRvIII
ABT-414
GFP+ 70.5
GFP- 29.3
GFP
Live/Dead Zombie NIR
B
ZH-161 GFP
control
GFP pos. 74.4
GFP neg. 24.7
ZH-161 GFP
ABT-414
GFP pos. 73.1
GFP neg. 25.9
ZH-161 EGFRvIII
control
GFP pos. 0.38
GFP neg. 99.5
ZH-161 EGFRvIII
ABT-414
GFP pos. 0.67
GFP neg. 99.2
ZH-161 GFP + EGFRvIII
control
GFP pos. 45.8
GFP neg. 53.4
ZH-161 GFP + EGFRvIII
ABT-414
GFP pos. 50.8
GFP neg. 48.4


C
%
150
100
50
0
LN-229 GFP
LN-229 EGFRvIII
Alive cells (untreated)
Alive cells (treated)
Dead cells
GFP ctr
GFP 10nM
EGFRvIII ctr
EGFRvIII 10nM
GFP/ EGFRvIII ctr
GFP/ EGFRvIII 10nM
GFP/ EGFRvIII ctr
GFP/ EGFRvIII 10nM

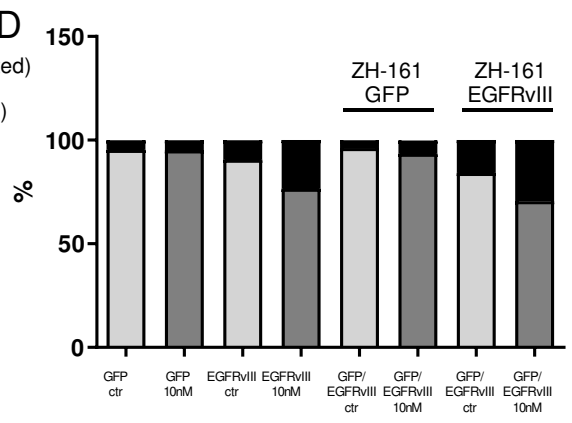

D
%
150
100
50
0
ZH-161 GFP
ZH-161 EGFRvIII
GFP ctr
GFP 10nM
EGFRvIII ctr
EGFRvIII 10nM
GFP/ EGFRvIII ctr
GFP/ EGFRvIII 10nM
GFP/ EGFRvIII ctr
GFP/ EGFRvIII 10nM

Figure 5

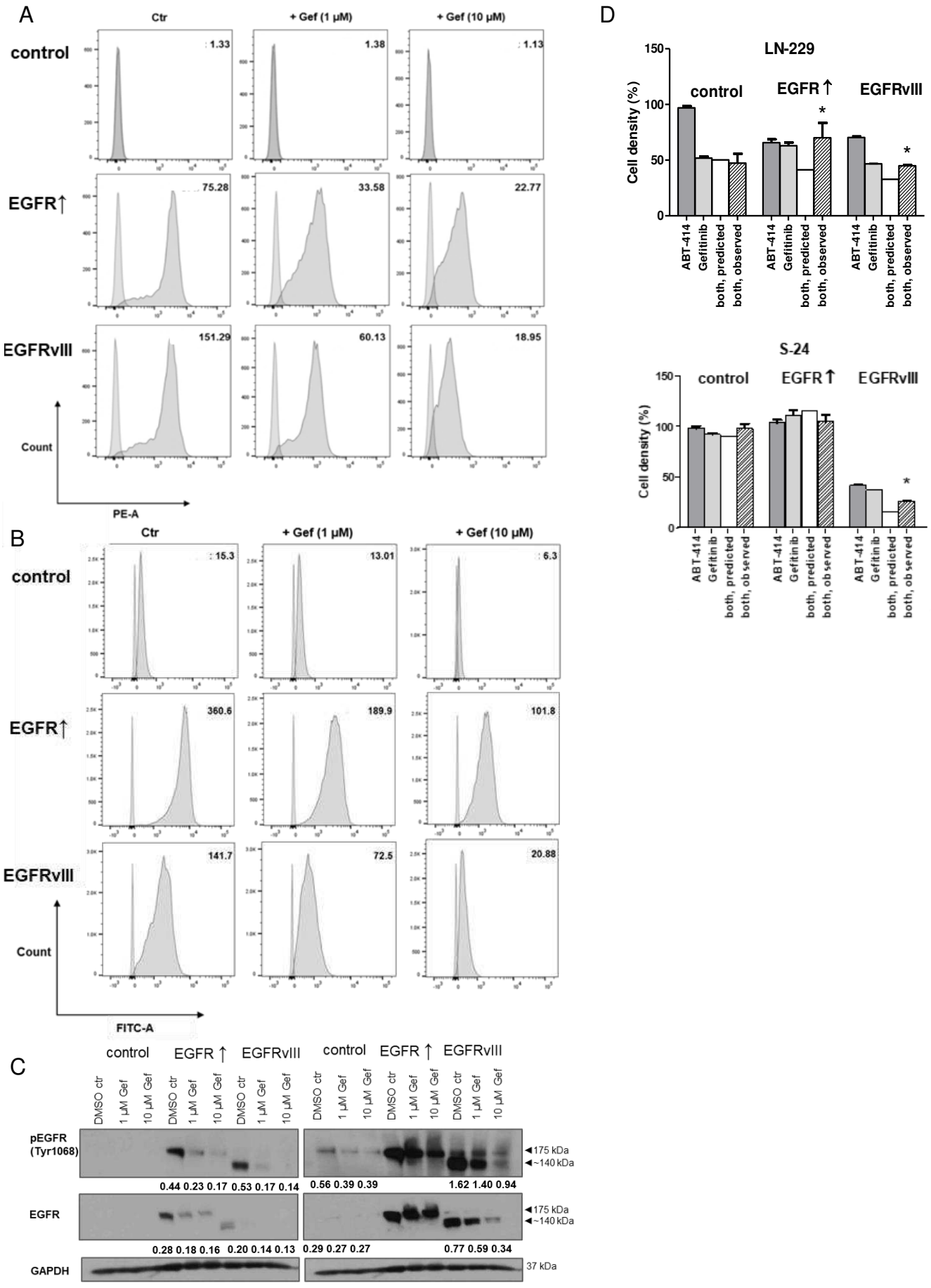
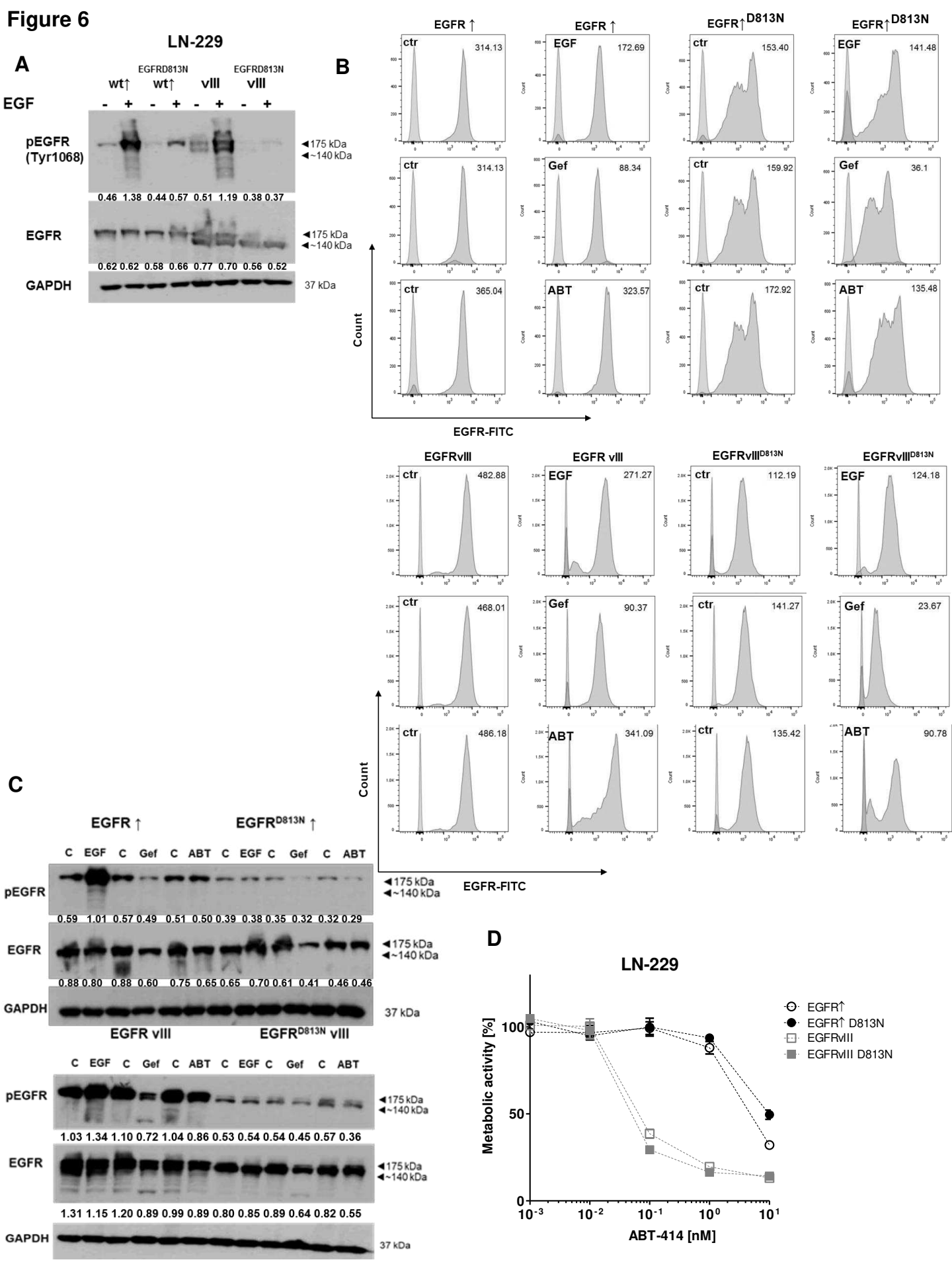

A
Ctr
+ Gef (1 μM)
+ Gef (10 μM)
control
EGFR↑
EGFRvIII
1.33
1.38
1.13
75.28
33.58
22.77
151.29
60.13
18.95
Count
PE-A
B
Ctr
+ Gef (1 μM)
+ Gef (10 μM)
control
EGFR↑
EGFRvIII
15.3
13.01
6.3
360.6
189.9
101.8
141.7
72.5
20.88
Count
FITC-A
C
control
EGFR ↑
EGFRvIII
control
EGFR ↑
EGFRvIII
DMSO ctr
1 μM Gef
10 μM Gef
pEGFR (Tyr1068)
175 kDa
~140 kDa
0.44 0.23 0.17 0.53 0.17 0.14
0.56 0.39 0.39
1.62 1.40 0.94
EGFR
0.28 0.18 0.16 0.20 0.14 0.13
0.29 0.27 0.27
0.77 0.59 0.34
GAPDH
37 kDa
D
LN-229
control
EGFR↑
EGFRvIII
Cell density (%)
150
100
50
0
ABT-414
Gefitinib
both, predicted
both, observed
S-24
control
EGFR↑
EGFRvIII
Cell density (%)
both, observed

Figure 6


LN-229
A
EGFRD813N
EGFRD813N
wt↑ wt↑ vIII vIII
EGF
- + - + - + - +
pEGFR (Tyr1068)
◄175 kDa
◄~140 kDa
0.46 1.38 0.44 0.57 0.51 1.19 0.38 0.37
EGFR
◄175 kDa
◄~140 kDa
0.62 0.62 0.58 0.66 0.77 0.70 0.56 0.52
GAPDH
37 kDa
B
EGFR ↑
EGFR ↑
EGFR↑D813N
EGFR↑D813N
ctr 314.13
EGF 172.69
ctr 153.40
EGF 141.48
ctr 314.13
Gef 88.34
ctr 159.92
Gef 36.1
ctr 365.04
ABT 323.57
ctr 172.92
ABT 135.48
Count
EGFR-FITC
EGFRvIII
EGFR vIII
EGFRvIII D813N
EGFRvIII D813N
ctr 482.88
EGF 271.27
ctr 112.19
EGF 124.18
ctr 468.01
Gef 90.37
ctr 141.27
Gef 23.67
ctr 486.18
ABT 341.09
ctr 135.42
ABT 90.78
Count
◄175 kDa
◄~140 kDa
EGFR-FITC
C
EGFR ↑
EGFRD813N ↑
C EGF C Gef C ABT C EGF C Gef C ABT
pEGFR
0.59 1.01 0.57 0.49 0.51 0.50 0.39 0.38 0.35 0.32 0.32 0.29
EGFR
◄175 kDa
◄~140 kDa
0.88 0.80 0.88 0.60 0.75 0.65 0.65 0.70 0.61 0.41 0.46 0.46
GAPDH
37 kDa
EGFR vIII
EGFRD813N vIII
C EGF C Gef C ABT C EGF C Gef C ABT
pEGFR
◄175 kDa
◄~140 kDa
1.03 1.34 1.10 0.72 1.04 0.86 0.53 0.54 0.54 0.45 0.57 0.36
EGFR
◄175 kDa
◄~140 kDa
1.31 1.15 1.20 0.89 0.99 0.89 0.80 0.85 0.89 0.64 0.82 0.55
GAPDH
37 kDa
D
LN-229
Metabolic activity [%]
100
50
0
10-3 10-2 10-1 100 101
ABT-414 [nM]
EGFR↑
EGFR↑ D813N
EGFRvIII
EGFRvIII D813N